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Cardiac progenitor cells in brown adipose tissue repaired damaged myocardium

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Abstract

Cardiomyocyte (CM) regeneration is limited in adult life and is not sufficient to prevent myocardial infarction. Hence, the identification of a useful source of CM progenitors is of great interest for possible use in regenerative therapy. Mesenchymal stem cells in bone marrow, embryonic stem cells, and skeletal myoblasts are known sources of CM repletion; however, there are a number of critical problems for clinical application. In this study, we succeeded to identify CM progenitor cells in brown adipose tissue (BAT). Moreover, we showed that CM progenitor cells in BAT that existed in CD29-positive population could differentiate into CM with high efficiency. To confirm the in vivo effect of $CD29^+$BAT-derived cells (BATDCs), we transplanted these cells into infarct border zone of an acute myocardial infarction model in rat. Results clearly indicated that implantation of $CD29^+$ BATDCs led to the reduction of the infarction area and improvement of left ventricular function by replacing newly developed CMs in comparison with that by $CD29^+$ white adipose tissue-derived cells or control saline. These findings suggest that BATDCs are one of the useful sources for a new strategy in CM regeneration.

Keywords: Regeneration; Heart; Myocardium; Adipose tissue

Cardiomyocytes (CMs) are thought to be terminally differentiated cells [1] and have been often compared to neurons for their inability to regenerate and replace damaged myocardium.

In recent years, work reported from several laboratories has helped to change this concept radically. Several investigators have succeeded in isolating and expanding neural stem cells that can generate neurons, oligodendrocytes, and astrocyte [2], as well as mesenchymal stem cells, which can give rise to different tissues such as bone [3,4,7], muscle [5], fat [6,7], and tendon or cartilage [7–9], suggesting the presence of one or more types of truly pluripotent stem cells. Even though there is much evidence associating adult organogenesis and multipotent stem cells, the concept of myocyte regeneration has not been embraced by the medical community and remains controversial. However, it has been shown that a small number of CMs retain the capacity to proliferate and regenerate in response to ischemic injury [10]. Replacement and regeneration of functional cardiac muscle after ischemic incidents to the heart could be achieved by either stimulating proliferation of endogenous mature CMs or resident cardiac stem cells ($c\text{-}kit^+$, $Sca\text{-}1^+$) [10–12], or by implanting exogenous donor-derived or allogenic cells such as embryonic stem cells [13,14], bone marrow-derived mesenchymal stem cells (MSCs) [15–18], skeletal myoblasts [19], or hematopoietic stem cells (HSCs) [20]. By employing these promising avenues of using stem cells, new therapies of myocardial infarction (MI) are now emerging. However, all of these therapies have some critical problems. The implantation of skeletal myoblasts

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has a risk of developing fatal arrhythmias, and in vivo evidence for functional cardiac improvement following transplantation of adult bone marrow-derived CMs has been exceedingly difficult to demonstrate to date [21,22]. Moreover, endogenous CMs or cardiac stem cells are not suitable for transplantation because of technical difficulties in collecting the cells. The clinical use of MSCs and HSCs has presented problems, including pain, morbidity, and low cell number upon harvest. Therefore, it is desirable to have an abundant source of CMs for use in humans.

Adipose tissue, like bone marrow, is derived from the embryonic mesoderm and contains a heterogeneous stromal cell population that is easily isolated [23]. Based on this, adipose tissue might represent a source of stem cells that could have far-reaching effects in several fields. Indeed, researchers have isolated adipose-derived stem cells (ADSCs) that possessed almost the same potential as MSCs and were able to differentiate into osteogenic, adipogenic, myogenic, and chondrogenic lineages when treated with established lineage-specific factors [24]. Moreover, it was reported that only a few cells derived from adipose stromal cells differentiate into beating cells that are thought to represent CMs phenotypically [25].

In the present study, we demonstrate that brown adipose tissue (BAT) contains a source of highly differentiating cardiac progenitor cells and these cells were found in higher abundance than in white adipose tissue (WAT). In addition, we found that CD29 is the stem/progenitor cell marker in BAT-derived cells (BATDCs). Finally, we showed that $CD29^{+}$ cells in BATDCs transplanted into ischemia-induced heart of rats differentiated into CMs, ECs, and SMCs, and reduced the infarction area and improved left ventricular function.

Materials and methods

Preparation and culture of cardiac progenitor cells. BAT was isolated from the interscapular and WAT was isolated from the inguinal of the neonate (post-coitum (P)1–7) or adult mice (8W). Isolated BAT and WAT were washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells. Then it was treated with 2.4 U/ml Dispase II (Roche, Mannheim, Germany) in PBS for 20 min at 37 °C with gentle agitation. Dispase II was inactivated with an equal volume of DMEM/10% fetal bovine serum (FBS). The samples were filtered through a 40-μm mesh filter (Falcon, Bedford, MA) to remove debris and were separated on Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) to select the mononuclear cells. We usually get $2–3 \times 10^5$ cells from one neonate. Then, samples were reacted with anti-CD31-PE (Pharmingen, San Diego, CA), and anti-PDGFR-β-PE (Pharmingen) and then reacted with anti-PE microbeads, and sorted negative fraction by Auto-MACS magnetic cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Next, cells were reacted with anti-CD29-biotin (Pharmingen), and streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach), and sorted the positive fraction by Auto-MACS (Miltenyi Biotec) obtaining purity of over 94%. Purified cells were plated onto tissue culture dishes in DMEM/10% FBS. In experiments involving limiting dilution of sorted BATDCs and WATDCs, we suspended 1×10^2 to 1×10^5 cells and cultured them on 24-well dishes (Nunc, Roskilde, Denmark) in DMEM/10% FBS. In both experiments, a half volume of growth medium was changed every three days until staining.

Immunohistochemistry. Immunohistochemical analyses on tissue sections and culture dishes were performed as previously described [26]. The tissue fixation procedures were the same as those described previously [26]. The fixed specimens were embedded in OCT compound and sectioned at 7 μm. An anti-vWF (Dako, Glostrup, Denmark), anti-SMA Cy3 (Sigma, St. Louis, USA), anti-sarcomeric actin (Sigma), anti-GATA-4 (Santa Cruz, CA, USA), anti-MEF2C (Cell signaling, Beverly, MA), anti-connexin43 (Sigma), anti-cardiac troponin-I and T (Santa Cruz), and anti-GFP biotin antibody (Santa Cruz) for tissue section, and anti-sarcomeric actin, anti-GATA-4, anti-SMA Cy3, anti-PDGFR-β (Pharmingen), anti-PECAM-1-PE (Pharmingen), and anti-VE-cadherin (Pharmingen) for culture dish were used in this assay. In brief, an anti-sarcomeric actin was developed with Alexa Fluor 488 or 546 or 633 conjugated goat anti-mouse IgM (Molecular Probes, Eugene, OR), and anti-MEF2C, anti-GATA-4, anti-connexin43, anti-cardiac troponin-T antibody were developed with Alexa Fluor 488 or 546 or 633 conjugated goat anti-rabbit IgG (Molecular Probes), and anti- PDGFR-β and anti-VE-cadherin were developed with Alexa Fluor 546-conjugated goat anti-rat IgG (Molecular Probes). Nuclear staining was performed with DAPI or TOPRO3 (Molecular Probes). Finally, the sections and dishes were observed and photographed under a microscope (IX-70, Olympus, Tokyo, Japan) with UV lamp or confocal microscope (LSM510, Zeiss, Germany).

Transmission electron microscopy. Cells were washed in phosphate buffer and fixed with 2% glutaraldehyde and 1% paraformaldehyde in PBS. Samples were postfixed with 1% osmium in PBS, rinsed, dehydrated, and embedded in araldite (Dako). Then, samples were cut with a diamond knife and examined under a Jeol 100CX electron microscope.

RT-PCR analysis. The RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) was used for isolation of total RNA from brown adipose tissues, cultured cardiac myocytes, and embryonic cardiac myocytes. Total RNA was reverse transcribed using the RT for PCR kit (Clontech, Palo Alto, CA). The cDNA was amplified using Advantage Polymerase Mix (Clontech) in a GeneAmp PCR system model 9700 (Perkin-Elmer Inc., Norwalk, CT) by 30–35 cycles. The sequences of the gene-specific primers for RT-PCR were as follows: 5′-α-MHC (TGTCTGCTCTCCACCGGG AAAATCT), 3′-α-MHC (CATGGCCAATTCTTGACTCCCATGA), 5′-β-MHC (AACCCACCCAAGTTCGACAAGATCG), 3′-β-MHC (CC AACTTTCCTGTTGCCCCAAAAATG), 5′-α-skeletal actin (GGAGAT TGTGCGCGACATCAAAGAG), 3′-α-skeletal actin (CTGGTTCCTCC AATGGGATATCTTC), 5′-α-cardiac actin (TGTGTTACGTCGCCCT GGATTTTGA), 3′-α-cardiac actin (TTGCTGATCCACATTTGCTGG AAGG), 5′-MLC-2a (AGCAGGCACAACGTGGCTCTTCTAA) 3′-MLC-2a (CCTGGGTCATGAGAAGCTGCTTGAA), 5′-MLC-2v (ATGGCACCTTTGTTTGCCAAGAAGC), 3′-MLC-2v (CCCTCGGG ATCAAACACCTTGAATG), 5′-GATA-4 (GAGTGAGTCAATTGTG GGGCCATGT), 3′-GATA-4 (TGCTGCTAGTGGCATTGCTGGAG TT), 5′-BNP (AAAAGTCGGAGGAAATGGCCCAGAG), 3′-BNP (TGCCTGAGGGGAAATGCTCAGAACT), 5′-NKx2.5 (CACACCCA CGCCTTTCTCAGTCAAA), 3′-NKx2.5 (CGACAGGTACCGCTGT TGCTTGAA), 5′-MyoD (TGCATTCCAACCCACAGAACCTTTG), 3′-MyoD (TCGAAGGCCTCATTCACTTTGCTCA), 5′-Myogenin (AG CGCAGGCTCAAGAAAGTGAATGA), 3′-Myogenin (ATGCACACC CAGCCTGACAGACAAT), and 5′-G3PDH (TGAAGGTCGGTGTG AACGGATTTGGC), 3′-G3PDH (CATGTAGGCCATGAGGTCCAC CAC). Each cycle consisted of denaturation at 94 °C for 30 s, and annealing/extension at 70 °C for 4 min.

Myocardial infarction and cell implantation and echocardiography. BAT and WAT derived from GFP transgenic Sprague–Dawley (SD) rats [27] were implanted. Myocardial infarction was produced in female SD rats at 2 months of age [10]. After making myocardial infarction, 10 rats were injected with 2×10^5 cells in five opposite regions bordering the infarct; they were sacrificed at 30 days. At each interval, sham-operated rats were injected with saline as controls. Under ketamine (Dainippon Pharmaceutical, Osaka, Japan) anesthesia, echocardiography was performed at 29 days. From M-mode tracings, LV end-diastolic diameter and wall thickness were obtained. Ejection fraction was computed. Echocardiographic acquisition and analysis were performed by an echocardiographer blinded to treatment group. Results represent means of five separate experiments.

Mortality was lower, but not significantly different, in treated rats, averaging 35% in all groups. Protocols were approved by the Institutional Review Board.

Action potential recording. Electrophysiological recordings were performed in IMDM (Sigma) containing (in mmol/L) $CaCl_2$ 1.48, KCl 4.32, and Hepes 25, (pH 7.4). Cultured cells were placed on the stage of an inverted microscope with phase-contrast optics (Nikon) at room temperature. Action potentials were recorded by conventional microelectrode. Intracellular recordings were made from 2 to 3 week cultured cells. Glass microelectrodes filled with KCl (3 mol/L) having a DC resistance of 15–30 MΩ were selected. Membrane potentials were measured by means of current-clamp mode (MEZ-8300; Nihon Kohden, Tokyo, Japan) with a built-in fourpole Bessel filter set at 1 kNz.

Pharmacological studies. The basal beating rate was recorded before addition of adrenergic reagents. Chronotropic responses were then assessed in DMEM/10% FCS by extracellular recording of the beating rate in the presence of the adrenergic reagents. Dose–response experiments were performed with 1 μM verapamil, $0.5–5 \times 10^{-6}$ mol/L isoproterenol, and $10–20 \times 10^{-6}$ mol/L propranolol. The values were presented as means ± SEM.

Results

Brown adipose tissue is a source of cardiomyocytes

There are two kinds of adipose tissues: the energy-storing WAT and the energy-dissipating BAT [28]. To test for the presence of stem cells and to find which adipose tissue contains cells capable of differentiating into CMs, we dissected BAT from the interscapular region and WAT from the inguinal region and isolated the mononuclear cells with Lymphoprep and separately cultured the cells on dishes in DMEM containing 10% FCS in appropriate cell density. Both cell preparations formed myotube-like structures and spontaneously began beating within one week of culturing. Synchronous beating was observed after one to two weeks in culture (data not shown); however, the number of beating cells in the culture derived from BAT was quite different to that derived from WAT. Furthermore, approximately 30–40% of beating cells existed in cells that thought to be cardiomyocytes morphologically, however, they were very few in WAT-derived CM. In order to further elucidate these findings, we performed immunohistochemical staining against sarcomeric actin (SA) and GATA-4 (Figs. 1Aa–d) and demonstrated that the number of CMs produced from BAT-derived cells (BATDCs) was 10 times higher than that of WAT-derived cells (WATDCs) (Table 1). BATDCs also differentiated into cardiac troponin-T-positive and MEF2C-positive cells (Figs. 1Ae and f). To enrich the CM progenitor cells, we selected CD29 as a marker, because CD29 was suggested to be one of MSC markers and progenitor/stem cell markers in several kinds of mesenchymal-derived cells, such as dermal cells. We sorted CD29-positive or negative cells

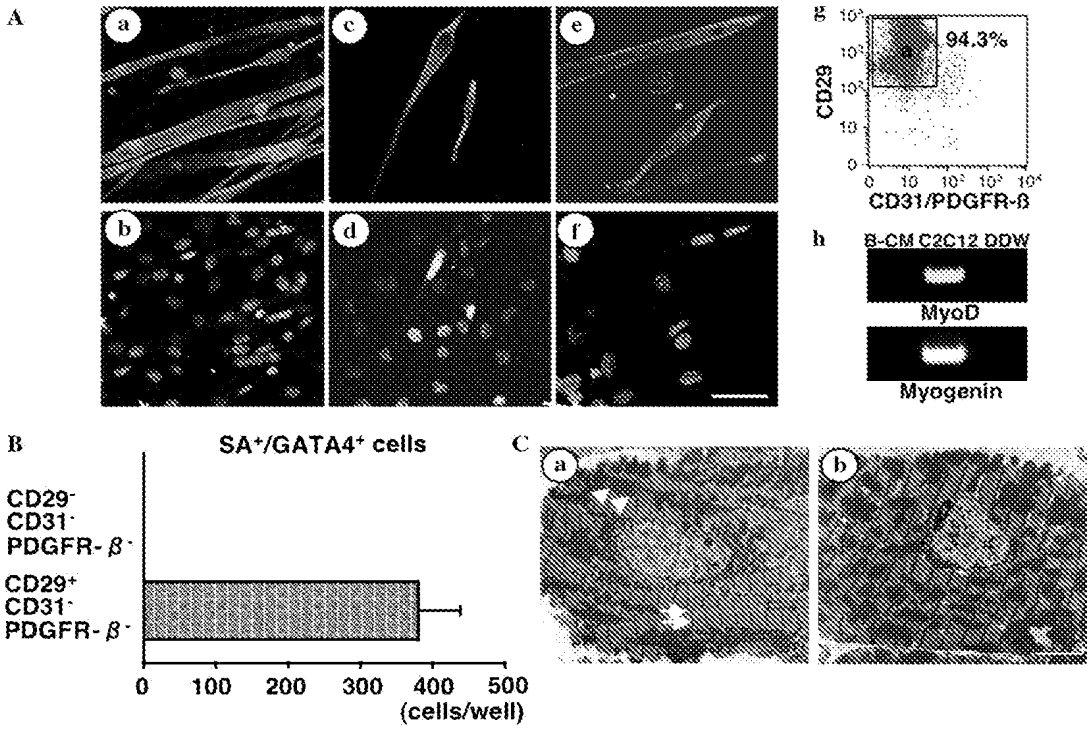

Fig. 1. The source of CMs in adipose tissue. (A) Cells derived from BAT and WAT were cultured for 14 days and then stained with anti-sarcomeric actin (SA) and anti-GATA-4 (a,c). For further confirmation, cardiac troponin-I and MEF2C staining was performed in BAT-derived CMs (e,f). Nuclear staining was performed with DAPI (b,d,f). Scale bar indicates 10 μm. (g) FACS analysis of $CD29^+CD31^-PDGFR\text{-}\beta^-$ cells after MACS sorting. Box indicated by (a) is sorting gate of $CD29^+$ cells. (h) RT-PCR analysis of BATCMs (B-CM) and C2C12 for their expression of MyoD or Myogenin. (B) The number of SA-positive/GATA-4-positive cells derived from $CD29^+$BATDCs or $CD29^-$BATDCs (mean ± SD, $n = 5$). (C) Transmission electron micrograph of BATCM (a) and neonatal CM (b). Of note, well-organized sarcomeres, *Z*-band (arrows) and a number of mitochondria (arrowheads) were observed in both BATCM (a) and neonatal CM (b). Scale bar indicates 2 μm.

Table 1
Appearance of $SA^+GATA\text{-}4^+CM$ from adipose tissues

Cell No.	1×10^5	5×10^4	1×10^4	5×10^3	2.5×10^3	1×10^3	1×10^2
BATDC (%)	18.6 ± 2.3	24.5 ± 2.3	20.5 ± 0.8	16.4 ± 0.6	5.8 ± 0.5	3.7 ± 0.2	—
WATDC (%)	2.1 ± 0.5	2.8 ± 0.3	1.5 ± 0.2	0.3 ± 0.1	0	0	0

CD29-positive cells from BAT and WAT as indicated were cultured for 14 days and percentage of $SA^+GATA\text{-}4^+$ cells in total adherent cells was determined. Data represent mean cell count ± SD ($n = 5$). *Abbreviations:* BATDC, brown adipose tissue-derived cell; WATDC, white adipose tissue-derived cell.

in $CD31^-PDGFR\text{-}\beta^-$ cells from BATDCs like Figs. 1Ag. These cells expressed CD34, CD90, and CD105 but did not express CD45 or CD106 (Supplemental data 1). 1×10^4 sorted cells were suspended on culture dishes. Nuclear staining with DAPI revealed that approximately 1000–1500 cells adhered to the bottom of dish. In adherent cells, 20–30% cells (200–450 cells per well) differentiated into $SA^+GATA\text{-}4^+$ CM. Surprisingly, we observed the SA and GATA-4-positive CM only from CD29-positive BATDCs (Fig. 1B). To further characterize BAT-derived CM (BATCM), we performed ultrastructural analysis of BATCMs to reveal the pattern of myofibrillar assembly and compared with normal CMs from neonatal mice. Electron microscopy revealed mononuclear cells of BATCM (Fig. 1Ca) with myofibrillar bundles oriented in a regular manner. Nuclei were in the center of the myotubes with multiple nucleoli and low chromatin content. The cytoplasm contained long mitochondria but few organelles. Cells contained myofibrils with the definitive ultrastructure of an organized sarcomere with typical cross-striation and developed *Z*-bands. The appearance of these cellular structures was quite similar to those of neonatal CMs (Fig. 1Cb). Moreover, the contracting cells did not express MyoD and Myogenin, which are a protein marker for skeletal muscles and are expressed on differentiated C2C12 cells (Fig. 1Ah). These observations indicated that BATDCs differentiated into CMs and CD29 was the marker for detecting CM progenitor cells in BATDCs. In the following experiments, we described CD29-positive cells in BATDCs as BATDCs.

The expression of CM-specific genes in BAT-derived cells

To further characterize cells displaying contractions, we verified the mRNA levels of cardiac-specific genes and transcription factors such as α-MHC, β-MHC, α-skeletal actin, α-cardiac actin, MLC-2a, MLC-2v, and BNP (Fig. 2A), and GATA-4 and NKx2.5 (Fig. 2B). Differentiated BATDCs expressed both the ANP (data not shown) and BNP genes. Both α- and β-MHC expression could be detected in BATCMs. BATCMs also expressed both α-skeletal actin and α-cardiac actin. Moreover, it was very interesting that BATCMs expressed MLC-2v, but not MLC-2a, suggesting that BATCMs had ventricle-like gene expression characteristics and not atrium-like gene expression [15].

As regards transcription-related genes, BATCMs expressed GATA-4, NKx2.5, and MEF2C (data not shown), while BATDCs did not express these CM-specific transcription factors (Fig. 2B).

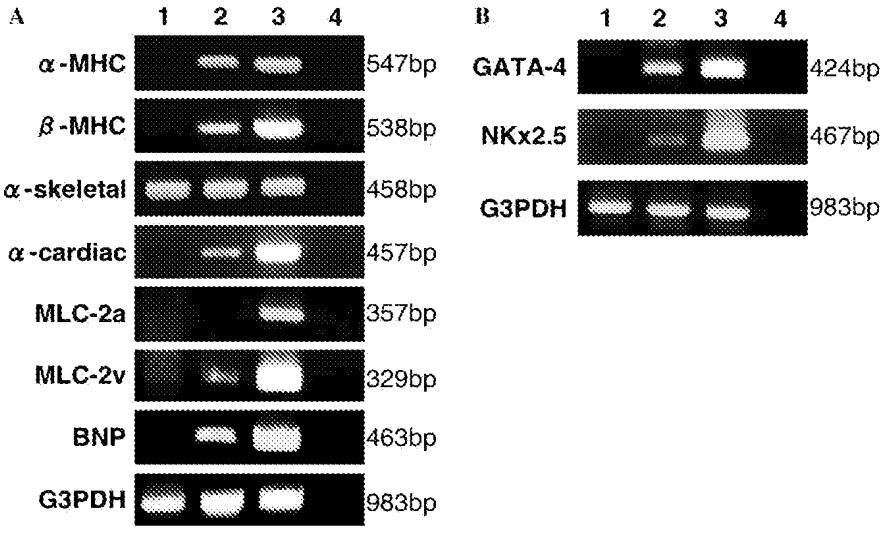

Fig. 2. RT-PCR analysis of CM-specific genes and transcriptional factors in BATCMs. Total RNA was isolated from mouse BAT (lane 1), BATCM (lane 2), embryonic CM (lane 3; as a positive control), and double-distilled water (lane 4; as a negative control). None of the CM-specific genes and transcriptional factors except the α-skeletal actin was observed in BAT before culturing. After 14 days of culturing, BATCMs expressed CM-specific genes and transcriptional factors except MLC-2a. The pattern of gene expression in the BATCMs corresponded to the phenotype specific to fetal ventricular CMs [15]. Results represent means of five separate experiments.

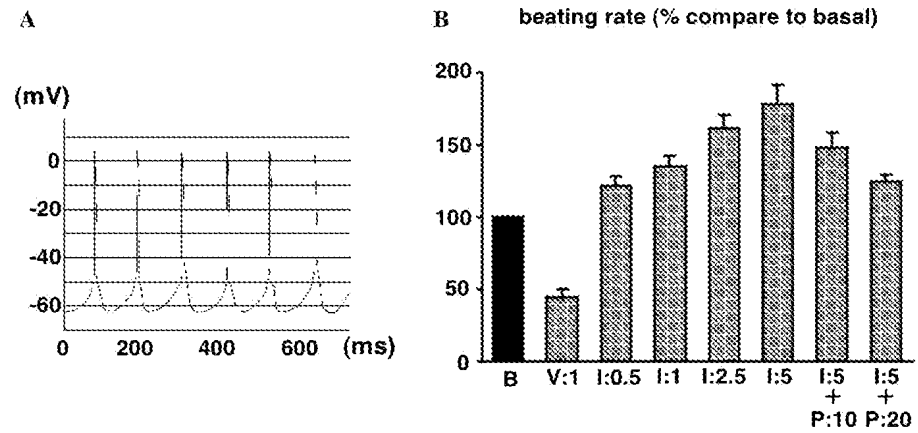

Fig. 3. The characteristic of BATCM as CM. (A) Electrophysiological analysis of contractile cells. Generated spontaneous action potential was recorded after application of a potential of −60 mV. Detail of six individual action potential was presented. (B) Chronotropic response of contracting cells to adrenergic stimulation. Contracting rate was measured under nontreatment (as basal beating rate: B) and treatment with Ca-antagonist verapamil (V), the β-adrenergic agonist isoproterenol (I) and antagonist propranolol (P). Results represent means of five separate experiments.

BATCMs have action potential and react with pharmacologic reagents

To provide additional evidence that BATCMs have the phenotype of CMs, we performed electrophysiological studies in BATCMs after 14 days of culturing. Cellular electrical activity was recorded on cells in current-clamp by using the whole cell configuration. BATCMs displayed a relatively shallow resting membrane potential with late diastolic slow depolarization, resembling a pacemaker potential (Fig. 3A). The calculated characteristics of this pace maker activity were as follows: beating cycle length = 122–360 ms, action potential duration = 49.6 ± 10.1 ms, action potential amplitude = 57.5 ± 10.6 mV, and maximum diastolic membrane potential = −56.5 ± 8.4 mV.

Next we checked the functionality of BATCMs by addition of several pharmacologic agents known to influence the heart rate. Pharmacological studies were performed at 28 days after culturing of BATDCs. The Ca-antagonist verapamil slowed the beating rate for BATCM and the β-agonist isoproterenol induced a dose-dependent increase of the spontaneous contraction rate for BATCMs; however, propranolol, which is a nonselective β-adrenergic antagonist, reversed the isoproterenol-induced acceleration (Fig. 3B). These findings indicated that BATCMs have the functional characteristics of CMs in vitro.

BATDCs differentiate into vascular cells as well as CMs

Recently, some sources for CMs such as multipotent MSCs, HSCs in BM, and c-kit or Sca-1-positive cells in the heart have been reported. These cells were also able to differentiate into vascular cells, such as endothelial cells (ECs) and smooth muscle cells (SMCs) in vitro. So we investigated whether BATDCs differentiate into ECs and SMCs in vitro. Fig. 4 clearly shows that SMA and PDGFR-β-positive cells or CD31 and VE-cadherin-positive cells were induced from BATDCs after 14 days of culturing. These indicated that both ECs and SMCs were generated from BATDCs in the same culture condition as above.

BAT-derived CMs regenerate functional myocardium in vivo

In order to determine whether BATCMs were capable of acquiring functional competence when properly stimulated in vivo, we tested their ability to differentiate and reconstitute the myocardium when injected into the hearts of rats following acute experimental MI by coronary ligation. To this end, 1×10^5 BATDCs were injected into the hearts of experimental MI rats at each of five sites at the infarct border area [10]. As control, experimental MI rats

Fig. 5. Repair of infarct with BATDCs in experimental MI in rats. (A) (a–d) Comparison of BATDC-injected group (a,c) with saline-injected group (b,d). The immunohistochemical analysis using anti-collagen type I and III antibodies in the areas of MI injected with BATDCs. Colors: collagen type I and III (blue), SA (red). Scale bar indicates 500 μm. (c,d) M-mode of echocardiography in BATDC-injected group (c) and saline-injected group (d). (e) Comparison of area containing collagen observed in (a,b). Percentage area in BATDC-injected hearts was calculated relative to saline, which was taken as 100%. Results represent means of five separate experiments. (f–k) Higher magnification of serial sections cut from the same area as in (a). (f–h) Nuclear staining with TOPRO3 (blue) (f), GATA-4 (green) (g), and SA staining (red) (h); image shown is merged with (f,g). (i–k) Nuclear staining with TOPRO3 (blue) (i), MEF2C (green) (j), and SA (red) (k); image shown is merged with (i,j). Scale bar indicates 15 μm. (B) To confirm whether newly appearing CMs were derived from implanted BATDCs, we performed immunohistochemistry with anti-GFP antibody. (a–c) GFP (green) (a), SA (blue) (b), and connexin43 (red) (c; arrows); image shown is merged with (a,b). (d–f) GFP (green) (d), cardiac troponin-T (red) (e), and nuclear staining with TOPRO3 (blue) (f); image shown is merged with (d,e). (g–l) Differentiation of BATDCs into vascular cells. (g–i) GFP (green) (g), vWF (red) (h); (i) shows merged images of (g,h). (j–l) GFP (green) (j), SMA (red) (k); (l) shows merged images of (j,k). Scale bar indicates 10 μm.

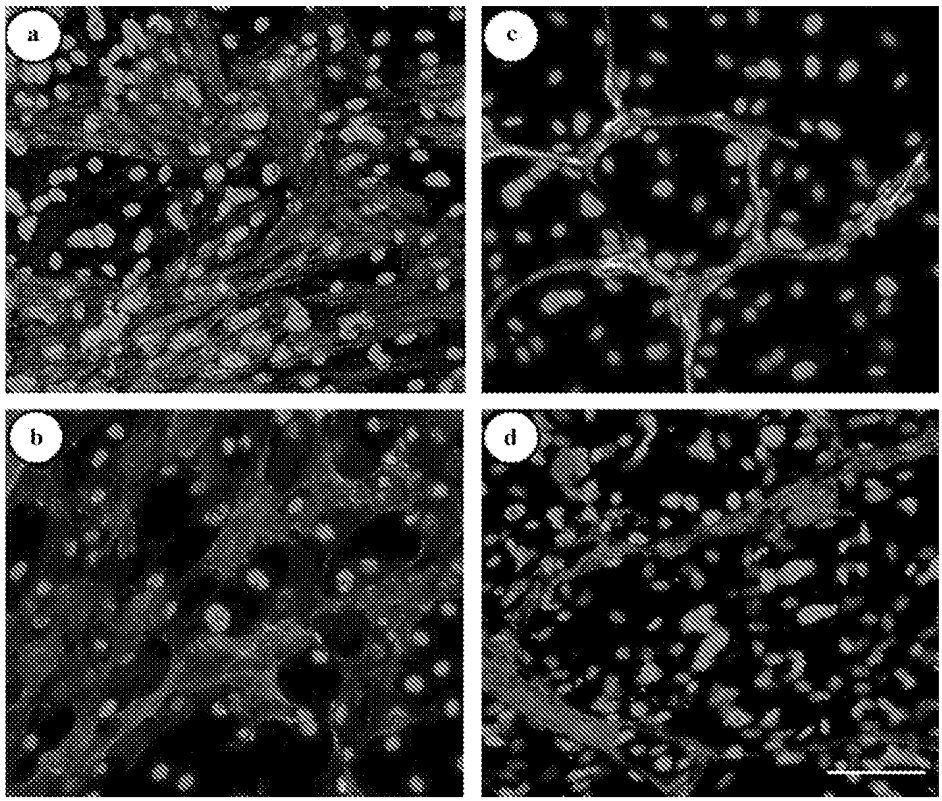

Fig. 4. Differentiation of BATDCs into ECs and SMCs. Selected BATDCs were cultured for 14 days and were stained with anti-αSMA (a), anti-PDGFR-β (b) for the detection of SMC development and anti-CD31 (c), anti-VE cadherin antibody (d) for the detection of ECs and DAPI (a–d) for the detection of nuclear. Scale bar in (d) indicates 25 μm.

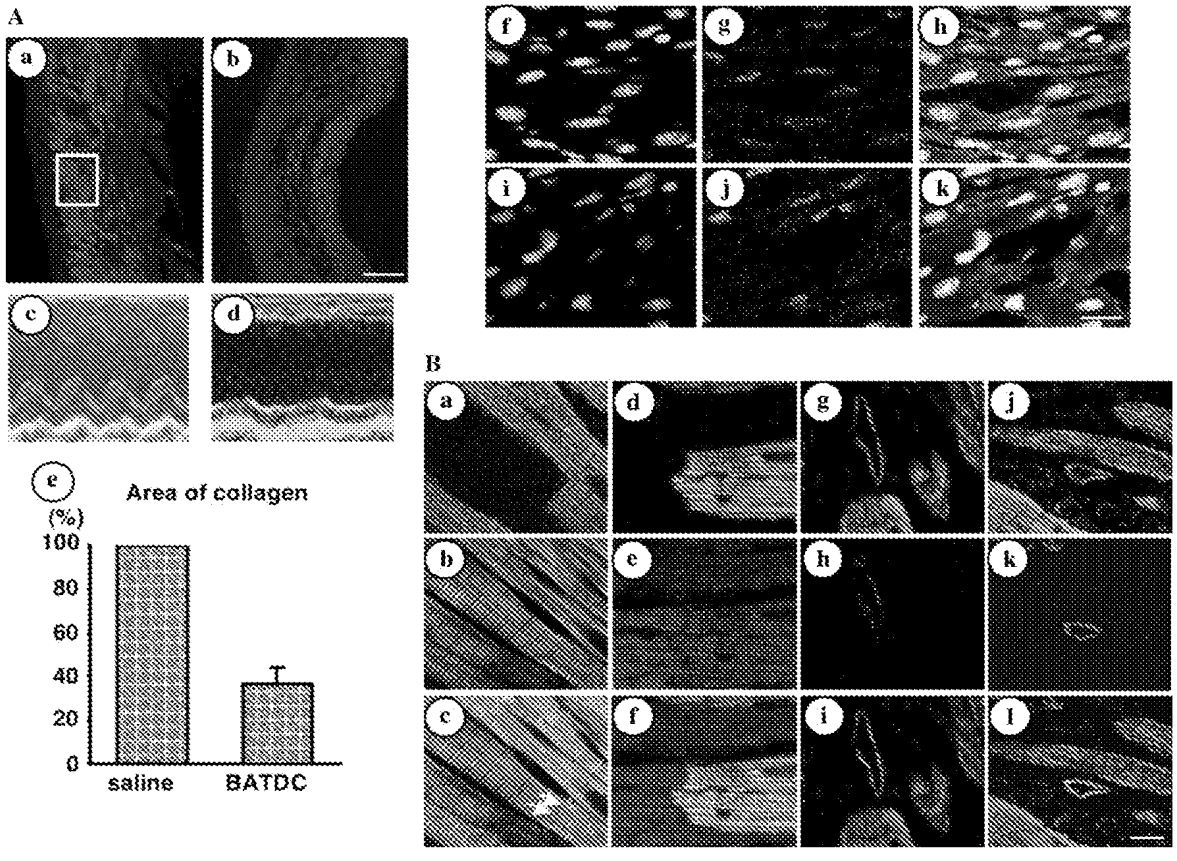

Table 2
Effect of transplanted cells on myocardial performance

	Sham	BATDC	WATDC	Saline
Ecocardiography				
Chamber diameter (mm)	6.03 ± 0.33	6.53 ± 0.41[a,b]	8.13 ± 0.48	10.3 ± 0.81
Viable WT (mm)	1.85 ± 0.18	1.78 ± 0.16[b]	1.71 ± 0.22	1.66 ± 0.13
Infarcted WT (mm)	1.8 ± 0.17	1.39 ± 0.25[a,b]	1.11 ± 0.35	0.75 ± 0.20
Ejection fraction (%)	95.6 ± 2.8	77.8 ± 6.1[a,b]	58.7 ± 7.5	37.5 ± 3.7

Echocardiography revealed that rats in $CD29^{+}$BATDC treatment group had significantly improved ejection fractions (EF) and reduced left ventricular diastolic diameters (LVIDd) compared with $CD29^{+}$WATDC treatment group or saline-treated group. Sham means sham operation.
Viable WT, viable wall thickness (not infracted wall), infarcted WT, infarcted wall thickness.
[a] Statistically significant difference from WATDC ($P < 0.01$).
[b] Statistically significant difference from saline ($P < 0.005$).

were injected with either equal volumes and number of WATDCs or with saline. At first, we stained the scarred area with anti-collagen type I and III antibodies to evaluate the remaining functional CM (Figs. 5Aa and b). The area containing collagen in the group implanted with BATDCs was reduced by about 70% compared with that of the saline-injected group (Fig. 5Ae). Direct assessment of cardiac function by echocardiography revealed that BATDC treatment group had significantly improved contractions movement of the infarcted anterior walls and reduced left ventricular remodeling compared with WATDC treatment group and saline group after coronary ligation (Figs. 5Ac and d and Table 2). Immunohistochemical staining indicated that injection of BATDCs led to the reconstitution of SA-positive and GATA-4-positive CMs (Figs. 5Af–h), or SA-positive and MEF2C-positive CMs (Figs. 5Ai–k). In order to evaluate the contribution of implanted BATDCs to CMs and vascular cells, we confirmed the expression of GFP in the implanted cells. First, we revealed that donor-derived GFP-positive and SA or cardiac troponin-T-positive cells were abundantly detected in the infarct border zone (Figs. 5Ba and d; 25.3 ± 3.4% of total ventricular cardiomyocytes in one field), but there were 1200-fold fewer after injecting CD29-negative cells in BATDCs (0.03 ± 0.01%). BATCMs also expressed CM-specific gene, such as connexin 43 (Fig. 5Bc: arrows), and cardiac troponin-T (Figs. 5Be and f). Moreover, a sub-population of GFP-positive cells expressed vWF which is an EC marker (Figs. 5Bg–i), and SMA which is a marker of SMCs (Figs. 5j–l), while infarcted hearts injected with saline as negative control did not show staining for any of these markers (data not shown).

Discussion

Recently, there has been great progress in the technique of revascularization by catheterization and coronary artery bypass grafting (CABG). However, ischemic heart disease continues to be a major cause of death as with cancer. Therefore, there is need for new therapies for the treatment of this intractable disease. Scientists have tried to establish methods for effective induction of multipotent stem cells including HSCs and MSCs to CMs or identification of stem cells that can differentiate into CMs. Moreover, effective strategies for the regeneration of heart tissue after MI have not yet been devised. To address these issues, we set out to identify a useful source of CMs and succeeded in identifying such a source of CMs in BAT.

In this study, we showed that CD29-positive cells in BATDCs were a more useful and enriched source of CMs than the previously reported cells, such as MSCs and HSCs. At first, we showed that BATDCs effectively produced CMs in vitro. A recent study showed that cells from adipose tissue differentiated into CMs in vitro [29]; however, the rate of cardiomyogenic differentiation was different from that in our study. This may be due to a difference in cell selection from adipose tissue and/or the culture conditions employed in our study and the previous study. In our case, we selected CD29-positive mononuclear cells in adipose tissues and cultured them in DMEM containing 10% FCS. This condition and appropriate cell density led more than 20% of the adherent cells to produce SA and GATA-4-positive CMs (Table 1). Moreover, these cells differentiated into ECs and SMCs. Probably, these ECs and SMCs supported the growth and differentiation of CMs, because inhibition of these cells by addition of neutralizing antibodies, such as anti-VEGFR-2 and anti-PDGF-β, inhibited CM differentiation in vitro (data not shown).

To clarify in vivo effect of BATDCs, we performed the transplantation to ischemic model rats. Transplantation of BATDCs into the infarct border zone restored up to 70% of the cardiac function compared with 95% in control sham operation (Table 2). By the difficulties of in vivo experiment using mice in our facility, we used rat in MI model. Therefore, it is difficult to compare cardiac repair rate between our experiment and others using mice. However, this cardiac repair rate was of a higher efficiency compared with that in previous reports [10,20,30]. Immunohistochemical analysis revealed that implanted cells effectively differentiated into ECs and SMCs as well as CMs. Furthermore, we did not observe the cell fusion mechanism between donor and host-derived cells in our experiments (unpublished data). Therefore, effective repair depends on the production of these three cell populations. Moreover, several reports have indicated that stromal cells derived from adipose tissue produce several angiogenic and anti-apoptotic factors, such

as VEGF, b-FGF, HGF, and TGF-β [31]. We also confirmed that BATDCs expressed VEGF, b-FGF, and HGF (data not shown). These factors might increase ECs, decrease TUNEL-positive apoptotic cells (data not shown), and inhibited the fibrosis (Fig. 5A). These contributed to effective cardiac repair in vivo.

Indeed, brown adipose tissue is very few in adult compared with infant, so our results do not directly connect with clinical application. Now we do not know the precise origin of brown adipocyte and white adipocyte and also those differentiation mechanisms. If we clarified them and found the critical factors of transdifferentiation from white adipose tissue to brown adipose tissue, our results might become very informative ones.

A study published by Orlic et al. [20] reported that HSCs in the adult BM transformed into myocardium and supported the development of vasculature. However, recently, data from several groups refuted transdifferentiation of HSCs into CMs in vivo [32–34]. Generally, plasticity of HSC is at present controversial; therefore, identification of a new source of CMs in BATDCs is a desirable outcome. Our results might shed light on this field by overcoming difficulties associated with CM regeneration.

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Appendix A. Supplementary data

Supplementary data associated with this article an be found, in the online version, at doi:10.1016/j.bbrc.2006.01.181.

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